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Evaluation of Chemical and Biological Methods for the Identification of Mutagenic and Cytotoxic Hazardous Waste Samples

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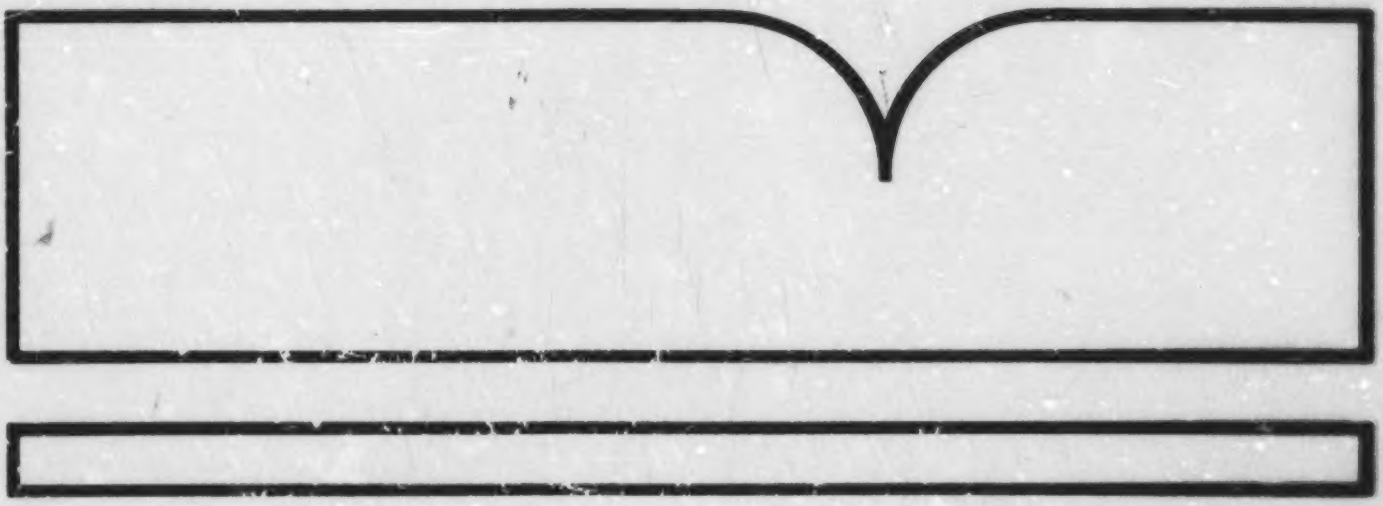

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THE EVALUATION OF CHEMICAL AND BIOLOGICAL METHODS FOR THE IDENTIFICATION OF MUTAGENIC AND CYTOTOXIC HAZARDOUS WASTE SAMPLES

by

Barbara Andon[1]
Marcus Jackson[1]
Virginia Houk[2]
Larry Claxton[3]

[1] Environmental Health Research and Testing, Inc.
P. O. Box 12199, Research Triangle Park, NC 27709

[2] Department of Environmental Sciences and Engineering
University of North Carolina, Chapel Hill, NC 27514

[3] USEPA, Health Effects Research Laboratory
Research Triangle Park, NC 27711

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NC 27711

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ABSTRACT

To assist in the development of methods for identifying potentially hazardous wastes, we have conducted studies on the extraction of toxicants from several solid waste samples. The extracts were tested for toxicity in the Chinese Hamster Ovary (CHO) Cytotoxicity Test and for mutagenic potential in the Salmonella Histidine Reversion Assay. A new technique was also employed which measured the mutagenicity of neat waste samples by coupling Thin Layer Chromatography (TLC) with the Salmonella Histidine Reversion Assay.

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Results showed that coke plant waste and herbicide mfg. acetone-water were mutagenic to S. typhimurium with the standard plate test. With the TLC technique, the neat coke plant waste was mutagenic and oil refining waste was

toxic. Oil refining waste was also toxic to CHO cells. Results of the gross chemistry determinations showed the three samples to have a wide range of solid content, total organic content, and extractables. Evaluation of the chemical extraction methods demonstrated few differences in extraction capabilities with respect to mutagenic activity.

KEY WORDS

Salmonella typhimurium
Chinese Hamster Ovary cells
hazardous wastes
mutagenicity
cytotoxicity
Thin Layer Chromatography

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INTRODUCTION

Short term *in vitro* cytotoxicity and mutagenicity assays have been used to predict the impact of pollutants on the environment and to support risk assessment procedures. Few *in vitro* studies, however, have been conducted to evaluate the biological activity which may be associated with industrial wastes. In the past the major research effort concerning wastes has been directed towards elucidating the toxicity associated with leachates[1]. Biological and chemical test methods have seldom been evaluated for use in detecting biologically active solid wastes.

In this study, the genotoxic and cytotoxic effects associated with three industrial waste samples have been determined. These samples were: coke plant waste (CPW), herbicide manufacturing waste acetone-water (HMW), and oil refining waste (ORW). The biological test methods employed were the Salmonella Histidine Reversion Assay and the Chinese Hamster Ovary Cytotoxicity Assay. Chemical methods involving the extraction of the three wastes with ethanol (EtOH), dichloromethane (DCM) and dimethylsulfoxide (DMSO) were evaluated. An additional technique, which coupled the chemical separation of wastes by Thin Layer Chromatography (TLC) with the Salmonella Histidine Reversion Assay, was also used.

This paper reviews the above methods for their ability to identify biologically active wastes samples.

MATERIALS AND METHODS

Waste samples

Coke plant waste, herbicide manufacturing waste acetone-water, and oil refining waste were the three hazardous waste samples (Table 1) evaluated in this study. The samples were obtained from Battelle Columbus Laboratories, Columbus, Ohio, courtesy of Dr. M. McKown. Samples were stored in the dark at 5°C in brown glass bottles with Teflon lined caps.

Gross chemical and physical characterization of waste samples

The following gross chemical and physical characteristics were determined for the three wastes: pH, density, total organic content, total solids, volatiles and semi-volatile content. Selected methods are presented below.

Organic extractables

The organic extractables were determined gravimetrically from the organic residue weight of a sample extract. A homogenized filtered sample was transferred to a separatory funnel and extracted three times with dichloromethane chloride. The extracts were combined and transferred to a Kuderna-Danish apparatus with a tared receiver. The extract was concentrated to 2 ml and then taken to dryness under a nitrogen stream. Each sample was subjected to aspirator vacuum for approximately 2 minutes to insure methylene chloride removal. The containers were reweighed to determine the absolute amount of organic extractables, which was reported as percent extractables (based on total sample weight).

Volatiles content

The approximate volatiles content of aqueous hazardous waste samples was determined using the modified EPA Method 601 (purge and trap analysis). The samples were analyzed with a Fischer-Victoreen gas chromatograph equipped with

flame ionization and Hall electrolytic conductivity detectors. A 1% SP-1000 packed column was used for analysis. Each sample (100 ul) was introduced into the purge vessel which contained 5 ml of prepurged distilled, deionized water. The sample was then purged following Method 601 procedures. The GC column effluent was split, with a portion of the stream passing to the flame ionization detector, and a portion passing to the Hall detector. All GC peaks were electronically integrated. Approximate quantitation was achieved by comparing the sample peak areas with peak areas of external standards in n-nonane and toluene for Flame Ionization Detector (FID); methyl chloroform for Hall Electric Conductivity Detector (HECD). Because the individual components of the samples were not known (and hence response factors were not known), the levels determined must be viewed as approximate.

Extraction of Waste Samples for Bioassay

Dichloromethane extracts (DCM). The sample was placed in a preweighed screw cap culture tube. After the sample weight was determined, a known volume of dichloromethane (DCM) was added to the tube. The tube was shaken to break up clumps, vortexed for 10 minutes, and centrifuged for 30 minutes. The DCM phase was withdrawn and filtered through a 5 ul Teflon filter into a capped vial. An aliquot (0.50 ml) of the extract was removed, blown to dryness under nitrogen gas, and the sample extract mass and concentration was determined. A known volume of sample extract was transferred to a clean vial and solvent exchanged into 1.0 ml dimethylsulfoxide for bioassay.

Ethanol extracts (EtOH). The above procedure was also employed for the ethanol extracts. However, no solvent exchange was performed since the solvent was compatable with bioassay procedures.

Dimethylsulfoxide extracts (DMSO). The same procedure for the DMSO extracts was used as for the DCM extracts except no solvent exchange was performed and no extract concentration was determined.

Bioassay methods

The Salmonella Histidine Reversion Assay. The S. typhimurium plate incorporation assay was performed as described by Ames et al.[2]. Most test samples were assayed twice with each of the five tester strains (TA1535, TA100, TA1537, TA1538, TA98) both with and without an Aroclor-1254-induced rat liver S9 homogenate at a concentration of 1.05 mg protein/plate. Triplicate plates at five test concentrations were evaluated. The five test concentrations employed resulted in a 4.5, 3.6, 2.7, 1.8 and 0.9% v/v supplement of the neat extract tc the bacterial cell suspension in agar. The 4.5% v/v supplement represented the maximum concentration of solvent which could be exposed to the bacteria before solven toxicity was observed. Criteria for a positive mutagenic response was based on the number of revertants for a treated culture equal to two times the background spontaneous value. In addition, the Stead et al.[3] program for the statistical evaluation of Ames test results was employed.

Thin Layer Chromatography/Ames technique. The TLC/Ames technique was a modification of the procedure developed by Bjorseth et al.[4,5] All samples were evaluated twice using tester strains TA98 and TA100 both with and without metabolic activation.

TLC separations were performed on commercially available glass backed cellulose plates (10cm x 10cm). Neat samples or extracts were spotted on the plates at an origin 1.5 cm from the bottom edge and 2.0 apart. Dose response relationships were determined by making four individual applications of increasing volumes (usually by a factor of 2) per plate. Spot diameters

were kept as small as possible to enhance resolution. The plates were developed at room temperature in chloroform for 15 to 20 minutes at which point the solvent front had ascended to approximately 1.0 cm from the top edge of the plate. After the plates were removed from the developing chamber and the mobile phase had evaporated, the plates were placed into petri dishes (150mm x 15mm). A mixture containing agar, minimal growth media, the bacterial strain, and the metabolizing system (if desired) was poured over the TLC plates. The petri dishes were incubated for 72 hours at 37°C. After incubation, the plates were examined for toxicity as evidenced by 1) a reduction in the overall number of revertants when compared to spontaneous values, or 2) a discrete toxic zone devoid of bacterial growth. A mutagenic event was recorded as a localized cluster of histidine revertants or as a profusion of revertants across the plate equal to two times the number observed on control plates.

The CHO Cytotoxicity Test. The samples were evaluated for cytotoxicity using the CHO cell system. Detailed aspects of analysis, sample handling, and culture and treatment of cells were performed as previously reported.[6] Briefly, CHO cells were added to individual wells of cluster dishes containing the waste extracts. Each extract was evaluated twice in triplicate at the maximum concentration obtainable with a 0.5% v/v supplement of extract. After a 20 hr exposure, cells were harvested and cell number and viability were determined simultaneously by exclusion of trypan blue using a hemocytometer. Adenosine-5-triphosphate (ATP) was assayed by luminescence using the luciferin - luciferase reaction.[7] Results from each determination were expressed as a percent of the control cell data, except for percent viability, and combined from replicate experiments. The viability index was determined by multiplying

the percent viability by the surviving fraction of cells for each treated culture. The viability index is an expression of cell viability that adjusts for any reduction in cell number due to toxic treatment. Any sample found to affect cell viability by depressing ATP generation below 50% of control was reevaluated in an effort to establish a dose response relationship.

RESULTS

The hazardous waste samples with their gross chemical parameters and physical descriptions are shown in Table 1.

The results of the mutagenic evaluation of the wastes with both the standard plate test and the TLC technique demonstrate that coke plant, herbicide manufacturing waste acetone-water, and oil refining wastes were mutagenic under one or several test conditions (Table 2). The model slope value is given for positive response with the standard plate test as an indication of the sample's relative potency. Since slope values cannot be determined with the TLC technique, a positive response is defined as a (+) for the TLC technique. A negative response is designated by a (-) for both assays. The concentration of the EtOH and DCM extracts utilized in the experiments are also listed. Due to the low vapor pressure of the solvent DMSO, no extract weights could be determined. As a result, the maximum volume of extract/plate utilized is listed. Where possible, density values were used to calculate the exposure concentration of neat samples in the TLC assay.

Coke plant waste demonstrated the greatest mutagenic activity with slope values ranging from 0.1 to 30. A positive mutagenic response with the coke sample was also detected with the TLC technique. The herbicide sample was the next most active sample with two slope values of 20. Oil refining waste was not mutagenic with the standard plate test but was detected with the TLC technique using the neat sample. This sample also demonstrated toxicity with the TLC technique in the absence of microsomal activation. In general, however, the addition of a rat liver metabolizing system enhanced the detection of mutagenic samples.

The results of evaluating a maximum (0.5% v/v) concentration of the waste extracts with CHO cells shows only the oil refining waste to be cytotoxic (Table 3). This sample produced a greater than 50% reduction in the viability index and cellular ATP content. A more definitive evaluation for dose response relationships, however, shows no parameter to be depressed greater than 50% (Figure 1). This may be a result of the sample's oily nature causing difficulty in delivering reproducible sample replicates and in its solubility.

Only the DMSO and DCM extracts are reported in Table 3 since the ethanol solvent proved to be cytotoxic at a 0.5% v/v concentration. Lowering the exposure volume to a nontoxic concentration of 0.01% v/v resulted in too low a sample concentration to warrant performing the experiments. In general, evaluation of the chemical extraction methods demonstrated few differences in extraction capabilities with respect to mutagenic activity.

DISCUSSION

Of the three samples tested coke plant waste was the most mutagenic. The activity of this sample resulted in plate counts that were 20 times greater than the spontaneous his^+ background values under certain test conditions (raw data not given). This observation is supported by the model slope values and the relatively low exposure concentrations. It was not surprising that this sample was mutagenic, since a GC/MS analysis (Table 4) performed by Battelle Columbus Laboratories showed it to contain 1.12 ug/g benzo(a)pyrene, 0.69ug/g benzo(b)- and benzo(k)fluoranthene, and 0.50 ug/g chrysene and benzo(a)anthracene.[8] Other investigators also have shown coke effluents to be mutagenic.[9]

Since identity of the actual herbicide(s) contained in the herbicide manufacturing sample is unknown, little can be said about the activity of this sample. GC/MS analysis (Table 5) revealed that 75 ug/g of the sample was an alkylamine(s) ($C_8H_{19}N$).[10] Chemical compounds containing this mass distribution are generally considered nonhazardous, however, the conversion of the amine group to a more active species is a possibility.

A limited amount of mutagenic activity was demonstrated for oil refining waste. This observation was not unexpected since a variety of organic bacterial mutagens, especially the polycyclic aromatic hydrocarbons (PAH) and alkylated PAH, are known to be associated with fossil fuels and their combustion effluents.[11] In addition, several reports defining the mutagenic activity of oil shale retort process water are present in the literature.[12,13]

Oil refining waste is currently subject to Resource Conservation and Recovery Act (RCRA) regulation because it has been shown to contain significant levels (in waste streams) of the toxic metals lead and chromium.[14] The Salmonella assay with the standard plate test or with the TLC technique does not detect the mutagenic or cytotoxic potential of metals.[15] Bioassay modifications and/or additional strains are required to enhance the sensitivity of the test for this class of chemicals. Only one metal, chromium, has been shown not to require alterations in assay parameters. As a result, the Salmonella assay may not be suitable for the routine mutagenic analysis of metal-containing wastes unless alternative experimental conditions are employed. For these reasons, it may be possible that the low levels of mutagenic and toxic activities observed with the neat sample may be some small percentage of the cumulative effects of these metal components under nonoptimal assay conditions.

The CHO cytotoxicity test, unlike the Salmonella assay, is sensitive to a variety of metallic compounds including lead and chromium salts.[16] This may explain the observed cytotoxic effects with oil refining waste in CHO. Unfortunately, no GC/MS data are available to aid in the analysis of the observed mutagenic and cytotoxic effects.

Each of the three assays employed in this study were capable of detecting the biological activity of the hazardous waste samples. There are, however, advantages and disadvantages associated with each system. The standard plate test, as a rule, is not capable of utilizing the neat complex mixture for routine analysis. Due to the incompatibility of the neat sample with the test system, extracts are employed. The TLC technique has the advantage of using neat samples. This technique is rapid, inexpensive, sensitive and may provide

a convenient method for prioritizing samples for additional testing. One disadvantage is the separation and detection of mutagenic chemical components depends greatly on the chemical characterisitics (solubility, polarity, charge) of the test sample and the mobile phase selected. An additional disadvantage is that the applied sample volumes must remain quite small, thus limiting the possibility of detecting weak mutagens present at low concentrations. The CHO system which is a more rapid technique than the bacterial test systems, may be useful in prioritizing samples for additional testing as well as aiding in identifying ranges of toxic potential. This system also has the advantage of being able to evaluate neat samples as well as extracts or even leachates. The major disadvantage of the CHO system is that it does not identify mixtures or compounds capable of causing heritable genetic damage.

Effective and inexpensive methods for evaluating the potential health effects of industrial process residuals are needed. These methods must utilize extraction techniques which reliably remove a representative population of chemical components from the waste sample. They must also be compatible with short-term in vitro assays capable of detecting genetic damage and cytotoxicity. The results presented here demonstrate that EtOH, DCM, and DMSO extraction of a variety of solid wastes does remove and allow detection of mutagenic and cytotoxic components. A useful addition to the screening procedure assay would be the use of bacterial strains capable of identifying wastes containing mutagenic metals. Future work will involve the characterization of additional classes of waste samples to further discriminate between test systems and chemical methods. Attempts will be made to identify the mutagenic and cytotoxic components of selected samples by coupling high performance liquid chromatography with suitable bioassays.

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Gross Chemical and Physical Characteristics of Waste Samples

Waste	Description	pH	Density	TOC[a] mg/l (mg/g)	Total Solids (%)	Volatiles (mg/ml)	Extractables (%)
Coke Plant Waste	Light brown fluid with suspended solids, 95% DMSO soluble, water insoluble	8.0	1.0	189	4.0	0.78	0.27
Herbicide Mfg. Acetone-Water	Red brown clear liquid, 100% DMSO soluble	6.5	1.1	20044	20.5	1.1	0.06
Oil Refining Waste	Dark liquid with brown black flocculent and oil droplets, DMSO and water soluble	7.0	NA[b]	(7.3)	58.5	NA	4.22

aTOC, total organic content.

bNA, not applicable.

Table 1

Results of the Mutagenic Activity of Waste Samples Using the *Salmonella typhimurium* Histidine Reversion Assay

Waste	Extract[a]	Concentration[b] µg (Volume) Extract Per Plate	Standard Plate Test										Concentration[d] µg (Volume) Per Plate	TLC Technique			
			TA100[c]		TA1535		TA1537		TA1538		TA98			TA98		TA100	
			+ S9	– S9	+ S9	– S9	+ S9	– S9	+ S9	– S9	+ S9	– S9		+ S9	– S9	+ S9	– S9
Coke Plant	Neat		NT[e]	NT	NT	NT	NT	NT	NT	NT	NT	NT	10	–, –	–[g]	–	–
	ETOH	50	10.2[f]	–[g]	–	–	0.9	0.1	6.9	0.3	30.0	–	3	+	–	–	–
	DCM	60	1.3	–	–	–	1.1	–	3.6	–	2.8	–	. . .	NT	NT	NT	NT
	DMSO	(100 µl)	1.4	–	–	–	0.8	1.4	–	–	15.0	–	(8 µl)	–	–	–	–
Herbicide Mfg. Acetone-Water	Neat		NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	–	–	–	–
	ETOH	4000	–	–	–	–	–	–	0.1	0.01	0.006	–	. . .	NT	NT	NT	NT
	DCM	10	–	–	–	–	20.7	–	11.6	2.4	19.9	–	. . .	NT	NT	NT	NT
	DMSO	(100 µl)	–	–	–	–	–	–	–	–	–	–	. . .	NT	NT	NT	NT
Oil Refining	Neat		NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	+	T[g]	–	–
	ETOH	2000	–	–	–	–	–	–	–	–	–	–	. . .	NT	NT	NT	NT
	DCM	16	–	–	–	–	–	–	–	–	–	–	. . .	NT	NT	NT	NT
	DMSO	(100 µl)	–	–	–	–	–	–	–	–	–	–	. . .	NT	NT	NT	NT

[a]Ethanol: ETOH, dichloromethane: DCM, dimethylsulfoxide: DMSO.

[b]Approximate concentration per plate: maximum volume per plate where weight of extract could not be determined.

[c]+ S9: experiment performed in the presence of Aroclor induced S9; – S9: experiment performed in the absence of Aroclor induced S9.

[d]Approximate concentrations derived from density values for neat samples.

[e]NT: not tested.

[f]Model slope values for positive responses with the standard plate test.

[g]–: negative response; +: positive response with the TLC technique; T: toxic response.

Table 2

Results of the Toxic Potential of Three Hazardous Waste Samples Using the CHO Cytotoxicity Test

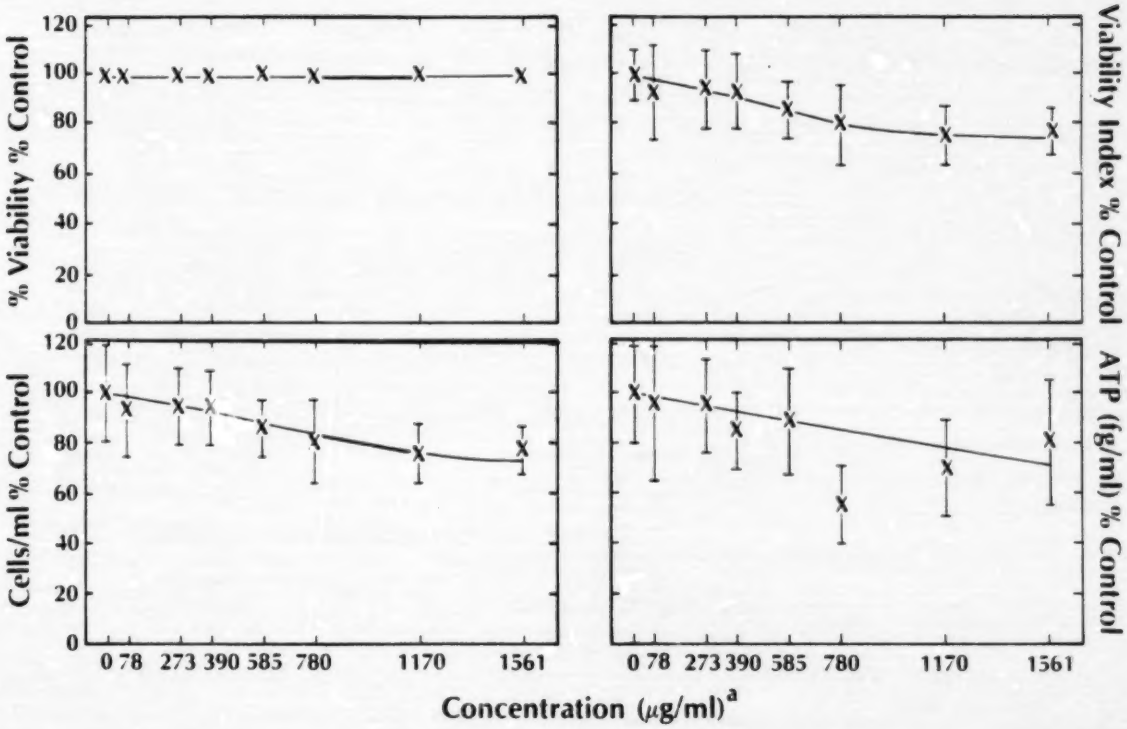
Waste	Extract[a]	Concentration µg/ml (% v/v)	% Viability	Viability Index[b]	Cell #/ml[b]	ATP (fg/ml)[b]
Coke Plant	DCM	6	97	107	111	87
	DMSO	(0.5)	99	99	100	108
Herbicide Mfg. Acetone-Water	DCM	1	98	111	114	93
	DMSO	(0.5)	99	93	94	95
Oil Refining	DCM	1560	68	43	63	24
	DMSO	(0.5)	78	74	78	78

[a]Dichloromethane, DCM; dimethylsulfoxide, DMSO.

[b]Expressed as a percentage of controls.

Table 3

Toxicity of Oil Refining Waste in the CHO Assay DCM Extracts (mean of 6 replicates)



[a]Approximate Concentrations

Figure 1

Results of GC-MS Analysis[a] of Coke Plant Waste

Compound	Coke Plant Waste (μg/g)
Purgeable Organics	
• Methylene chloride	0.09
• Trichloroethylene	0.00
• Benzene	0.00
• 1,1,2,2-Tetrachloroethene	0.00
• 1,1,2,2-Tetrachloroethane	0.00
• Toluene	0.00
• Chlorobenzene	0.00
• Ethylbenzene	0.00
Base/Neutral Extractable Semivolatile Organics	
• Naphthalene	0.00
• Diethyl phthalate	0.00
• Di-n-butyl phtalate	0.00
• Bis(2-ethylhexyl)phthalate	1.49
• Chrysene/benzo(a)anthracene	<0.50
• Benzo(b)/benzo(k)fluoranthanes	0.69
• Benzo(a)pyrene	1.12
Acid Extractable Semivolatile Organics	
• 2-chlorophenol	0.00
• Phenol	<0.50
• Pentachlorophenol	2.17

[a]Analysis performed by Battelle Columbus Laboratories.

Table 4

Results of GC-MS Analysis[a] of Herbicide Mfg. Acetone-Water

Compound	μg/g
Alkylamine ($C_8H_{19}N$)	75
Alkylamine	9
Alkyl ketone (MW = 128)	8
Unknown	20
Unknown	30
Unknown	9

[a]Analysis performed by Battelle Columbus Laboratories.

Table 5